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TGF-β signaling in insects regulates metamorphosis via juvenile hormone biosynthesis

Short title: Regulation of JH biosynthesis by TGF-β signaling

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Footnotes

Author contributions

Y.I., T.M., and S.N. designed this study; T.M. and S.N. contributed equally to this study; Y.I., T.W., and T.B. performed the cDNA cloning; Y.I. performed all of the RNAi, qPCR, and hormonal treatment experiments; K.T., Y.I., and Y.M. performed the *in situ* hybridization; Y.I. and S.T. performed the LC-MS; Y.I., K.M., T.M., H.O., and S.N. analyzed the data; Y.I and S.N. wrote the main manuscript; and all of the co-authors contributed to the discussion of results.

Author Conflict of Interest

The authors declare no conflict of interest.

Abstract

While butterflies undergo a dramatic morphological transformation from larvae to adult via a pupal stage (e.g., holometamorphosis), crickets undergo a metamorphosis from nymph to adult without formation of a pupa (e.g., hemimetamorphosis). Despite these differences, both processes are regulated by common mechanisms that involve 20-hydroxyecdysone (20E) and juvenile hormone (JH). JH regulates many aspects of insect physiology, such as development, reproduction, diapauses, and metamorphosis. Consequently, strict regulation of JH levels is crucial throughout an insect's life cycle. However, it remains unclear how JH synthesis is regulated. Here, we report that in the corpora allata (CA) of the cricket, *Gryllus bimaculatus* (*Gb*), Myoglianin (*Gb*'Myo), a homolog of *Drosophila* Myoglianin/vertebrate GDF8/11, is involved in the down-regulation of JH production by suppressing expression of a gene encoding JH acid *O*-methyltransferase, *Gb'jhamt*. In contrast, JH production is up-regulated by Decapentaplegic (*Gb*'Dpp) and Glass bottom boat/60A (*Gb*'Gbb) signaling that occurs as part of the transcriptional activation of *Gb'jhamt*. *Gb*'Myo defines the nature of each developmental transition by regulating JH titre and the interactions between JH and 20E. When *Gb'myo* expression is suppressed, activation of *Gb'jhamt* expression and secretion of 20E induces molting, thereby leading to the next instar prior to the last nymphal instar. Conversely, high *Gb'myo* expression induces metamorphosis during the last nymphal instar due to cessation of JH synthesis. *Gb'myo* also regulates final insect size. Since Myoglianin/GDF8/11 and Dpp/BMP2/4-Gbb/BMP5-8 are conserved in both

invertebrates and vertebrates, the present findings provide common regulatory mechanisms regarding endocrine control of animal development.

Significance Statement

Insects undergo a morphological transformation from nymph/larvae to adult with or without pupal formation, and these processes are referred to as hemi- and holo-metamorphosis, respectively. Both processes are regulated by common mechanisms involving the hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). However, it remains unclear how synthesis of JH is regulated in the corpora allata (CA). Here, we report that the TGF-β ligands, *Gb*'Myo (GDF8/11) and *Gb*'Dpp/Gbb, regulate JH synthesis via JH acid *O*-methyltransferase (*Gb*'*jhamt*) expression in the CA. Furthermore, loss of *Gb*'Myo function preserves the 'status quo' action of JH and prevents metamorphosis. These findings elucidate the regulatory mechanisms that provide endocrine control of insect life cycles, and also provide a new model of GDF8/11 function.

/body

Introduction

Holometabolous insects, such as butterflies, beetles, and flies, undergo a dramatic morphological transformation from larva to pupa to adult, and this process is referred to as holometamorphosis. Hemimetabolous insects, such as locusts, cockroaches, and crickets, also undergo morphogenesis to form mature wings and external genitalia, similar to that observed in the larva-to-pupa transition and pupa of holometabolous insects. However, the change of form is not drastic, given that nymphs are similar to their adult form. Despite these differences in metamorphic type, both hemimetabolous and holometabolous processes are regulated by common mechanisms involving the molting steroid, 20-hydroxyecdysone (20E), and the sesquiterpenoid, juvenile hormone (JH) (1-3). The latter regulates many aspects of insect physiology, such as development, reproduction, diapauses, and metamorphosis (4, 5). Consequently, strict regulation of JH levels is crucial throughout an insect's life cycle. JH is synthesized in and released from the corpora allata (CA), a pair of epithelial endocrine glands in the head (6-8). It has been hypothesized that JH biosynthesis is regulated by both stimulatory (allatotropic) and inhibitory (allatostatic) neuropeptides, and JH is able to reach the glands via the hemolymph and/or nervous connections (9). However, the mechanism(s) regulating JH synthesis remain unclear.

Temporal transcriptional control of *jhamt*, a gene that encodes a JH acid *O*-methyltransferase that converts inactive JH precursors into active JH, is thought to be critical for regulating JH synthesis (3, 10). Furthermore, the protein, JHAMT, has been

found to catalyze the final step of the JH biosynthesis pathway in the CA of various insects, including *Drosophila melanogaster*, *Tribolium castaneum*, *Apis mellifera*, and *Bombyx mori* (11-14). It has also been demonstrated that *jhamt* is predominantly expressed in the CA, and its developmental expression profile highly correlates with changes in JH titre. However, the molecular mechanisms underlying regulation of the temporal expression profile of *jhamt* remain unknown, and this represents a long-standing area of research in entomology (10). In order to elucidate the mechanisms underlying the regulation of JH titre, the cricket, *Gryllus bimaculatus* (15, 16), was employed as a model system of hemimetabolous ancestors that evolved into holometabolous insects (2, 17). In the present study, we demonstrate that *Gryllus bimaculatus* Myoglianin (*Gb*'Myo), a homolog of *Drosophila* Myoglianin (18)/vertebrate GDF8/11 (19), suppresses expression of *Gb'jhamt* in the CA of the cricket *Gryllus bimaculatus* to down-regulate JH production. Conversely, up-regulation of JH is achieved by *Gb*'Dpp and *Gb*'Gbb, members of the TGF-β family, as part of a signaling pathway that mediates transcriptional activation of *Gb'jhamt*. Overall, these findings provide a paradigm with which to better understand endocrine control of invertebrate developmental processes.

Results

In *Drosophila melanogaster* (*Dm*), it was reported that loss of *Dm*'*mad*, *Dm*'*tkv*, or *Dm*'*dpp* caused precocious metamorphosis, even in the early larval stages (20). Therefore, we first examined whether Dpp signaling plays a role in regulating *Gryllus* metamorphosis. For these studies, interfering RNA (RNAi) targeting *Gb*'*mad*, *Gb*'*tkv*, and Common mediator (Co)-Smad (*Gb*'*medea*) were individually injected into 3rd instar nymphs. The nymphs that received RNAi targeting *Gb*'*mad* or *Gb*'*tkv* achieved adult metamorphosis at the 7th instar rather than the 8th instar in both sexes (Fig. 1*A*; male n = 12/15, female n = 14/16 for *Gb*'*mad* and Fig. 1*B*; male n = 10/12, female n = 12/15 for *Gb*'*tkv*). In addition, an overall reduction in body size and weight were observed for both RNAi-treated nymphs (Fig. 1 *C* and *D*). Following the injection of RNAi targeting *Gb*'*mad,* dysgenesis of the wing pads (Fig. 1 *E* and *G*) and ovipositor (Fig. 1 *F* and *H*) were observed during the 6th instar and the precocious adult stage. Finally, RNAi-mediated depletion of *Gb*'*medea* led to precocious adult metamorphosis that occurred at the 7th instar (n = 10/21). As a result, malformation of the wing pads (Fig. S1 *D* and *E*) and ovipositor (Fig. S1*F*) were observed compared with the *DsRed2* RNAi control nymphs (Fig. S1 *A*-*C*; n = 31).

On the other hand, we identified three different *Gryllus* BMP homologs. Therefore, each of these homologs were targeted with RNAi, including: *Gb*'*dpp* (BMP2/4 homolog; n = 33), *Gb*'*dpp* like1 (BMP2-like homolog; n = 29), and *Gb*'*dpp* like2 (BMP3 homolog; n = 31). Combinations of these homologs were also targeted: *Gb*'*dpp* + *Gb*'*dpp* like1 (n = 25), *Gb*'*dpp* + *Gb*'*dpp* like2 (n = 28), *Gb*'*dpp* like1 +

Gb'dpp like2 (n = 27), and *Gb'dpp* + *Gb'dpp* like1 + *Gb'dpp* like2 (n = 28). However, all of the nymphs that received these RNAi treatments developed normally to become adults (Fig. S1*M*). It is possible that the absence of an effect in these experiments may be due to the presence of other redundant ligand(s).

To identify ligand(s) that may be redundant for *Gb'dpp*, RNAi was next used to target various *Gryllus* homologs of the *Drosophila* TGF-β family members (21), including: *glass bottom boat/60A* (*Gb'gbb*), *activinβ* (*Gb'actβ*), *maverick* (*Gb'mav*), and *myoglianin* (*Gb'myo*).

Initially, it was investigated whether depletion of *Gb'gbb* mRNA could be linked to the effects associated with loss of *Gb'tkv, Gb'mad*, or *Gb'medea*. Following the injection of RNAi targeting *Gb'gbb* into 3rd instar nymphs, precocious differentiation of adult features was observed, and these features were similar to those exhibited by the nymphs that underwent depletion of *Gb'mad* or *Gb'medea* by RNAi. However, the number of *Gb'gbb*-depleted nymphs that were obtained was substantially lower (Fig. S1 *G-I*; n = 6/33). Since Gbb forms a heterodimeric complex with Dpp in *Drosophila* (22-24), we hypothesized that the simultaneous depletion of *Gb'dpp* and *Gb'gbb* would be sufficient to impair normal adult development, especially in the wing pads and ovipositor. Therefore, we next injected RNAi targeting *Gb'dpp* and RNAi targeting *Gb'gbb* into 3rd instar nymphs. A total of 14/16 nymphs exhibited precocious adult metamorphosis. Furthermore, the wing pads and ovipositors of the resulting 6th instar nymphs and precocious adults resembled those of the nymphs that received RNAi targeting *Gb'mad* or *Gb'medea* (Fig. S1 *J-L*). In contrast, the combination of targeting

Gb'dpp like1/2 and *Gb'gbb* with RNAi did not affected the ratio of appearance of precocious adult metamorphosis (*Gb'dpp* like1 +*Gb'gbb*; n = 2/15 and *Gb'dpp* like2 + *Gb'gbb*; n = 4/17). Overall, these results demonstrate that the Dpp signaling pathway is triggered by heterodimeric ligand complexes of Dpp and Gbb, and Dpp/Gbb signaling via Tkv and Mad/Medea is critical for ensuring the completion of adult metamorphosis.

When RNAi targeting *myoglianin* was injected into 3rd instar nymphs, a supernumerary nymphal molt was observed in 52/59 of the injected nymphs. In comparison, the nymphs that were injected with RNAi targeting *DsRed2* (the control nymphs; n = 33) underwent normal molting between the 4th and 8th instars and then became adults (Figs. 2*A* and S4*N*). The molting of the *myoglianin*-targeted nymphs specifically involved a progression series of 3rd-3'-3''-4th-4'-4''-5th or 3rd–3'-4th-4'-4''-4'''-5th (instead of 3rd-4th-5th), and then they subsequently underwent a 6th instar molt (Fig. 2 *A* and *B*). We subsequently identified the *myoglianin* homolog as, metamorphosis inducing factor (*Gb'myo*), and its predicted amino acid sequence contains hallmarks of the TGF-β family members (Fig. S2 *A*-*C*). Moreover, although the nymphs injected with RNAi targeting *Gb'myo* blocked the morphological transition from one nymphal instar to the next, the number of supernumerary molts at each instar was restricted to 1–3 molts. Moreover, when the 6th instar nymphs became adults after these supernumerary molts, their body size and weight were significantly greater than those of the controls (Fig. 2 *B*, *T,* and *U*), and the developmental period for metamorphosis was approximately twice that of the controls (Fig. S4*N*). However, progressive morphogenesis of the wing pads (Fig. 2 *G*-*K*) and the ovipositor primordial

(Fig. 2 *P-S*) remained unchanged in the supernumerary nymphs over an extended period of time, whereas the control nymphs developed normal wing pads (Fig. 2 *C-F*) and ovipositors (Fig. 2 *L-O*). Furthermore, when RNAi targeting the TGF-β signaling factor *smox*/*Smad2* (*Gb'smox*) (Fig. S3 *A*, *D,* and *E*; n = 17/22) and RNAi targeting the type I receptor *baboon* (*Gb'babo*) (Fig. S3*B*; n = 10/15) were injected into 3rd instar nymphs, similar phenotypes as those associated with the control nymphs were observed. Based on these RNAi results, targeting of *Gb'myo*, *Gb'babo*, and *Gb'smox* appears to preserve the 'status quo'; and then after molting, wings and ovipositors are able to form normally – potentially due to loss of the RNAi effects.

To further investigate the 'status quo' preservation that characterized the RNAi targeting of *Gb'myo*, the same RNAi was injected into 4th instar (Fig. S4 *A* and *B*, Fig. S4*C*; n = 15/15 for a second dose of RNAi targeting *Gb'myo* at the 5th instar), into 5th instar (Fig. S4 *D, G,* and *H*; n = 14/15), and into 6th instar (Fig. S4 *K* and *L*; n = 10/10) nymphs within the first 24 h after ecdysis. Changes in the wing pads and ovipositor for these stages (Fig. S4 *E-L*), in the relative amounts of *Gb'myo* transcripts (Fig. S4*M*), and the temporal profile of these changes (Fig. S4*N*) suggest that *Gb'myo* may determine the molting characteristics that occur between different nymphal instars. Furthermore, loss of the functions mediated by the *Gb*'Myo protein resulted in developmental arrest and death at the 6th instar.

Methoprene is an analog of JH and it was also applied to nymphs during the 3rd instar. Following this treatment, supernumerary molting occurred and larger adults resulted (Fig. S3 *C-E*; n = 17/23). A similar phenotype was observed for the nymphs

that received *Gb'myo*-targeted RNAi. Therefore, we hypothesized that the latter might be due to a constant JH titre.

To examine a potential dependence of nymphal instars on the concentration of JH in the hemolymph, JH III production was monitored. Periodic changes in JH III production were observed (Fig. 3*A*), and at the final (8^{th}) instar, the titre of JH III declined to a low level on day 1 and then was not synthesized until day 7 to allow for adult molting (Fig. 3*A*). To further examine whether periodic changes in the JH III titre depended on *Gb*'Myo function, JH III titres were quantified on day 5 for the supernumerary instars (3' and 4^{th}) that had received RNAi targeting *Gb'myo*. Loss of *Gb'myo* mRNA resulted in constitutively higher JH III titre levels, whereas introduction of RNAi targeting *Gb'mad* only lowered the JH III titre levels on day 1 of the 4^{th} and 6^{th} instars (Fig. 3*B*). In combination, these data suggest that *Gb*'Mad and *Gb*'Myo play crucial roles in controlling JH biosynthesis.

To investigate the spatial and temporal expression patterns of *Gb'myo* mRNA, quantitative RT-PCR (qPCR) was performed. *Gb'myo* mRNA was found to be highly expressed in the head and thorax 1 (Fig. S5*A*), while the levels of *Gb'myo* mRNA exhibited periodic changes in each of the instars, with a peak in *Gb'myo* mRNA detected on day 3 (Fig. 3*C*). While a stepwise increase in the levels of *Gb'myo* mRNA was observed throughout the developmental stages, they were not observed in adulthood. Moreover, the levels of *Gb'myo* mRNA exhibited no obvious differences between males and females during all nymphal and adult stages. When the levels of *Gb'jhamt* mRNA were detected, peaks in expression were initially observed on day 1 in

each instar, they decreased by day 3, and then they completely disappeared on the day before molting (Fig. 3*E*). This pattern may be associated with the ecdysis process which is closely tied to the JH cycle. In contrast, *Gb'dpp* mRNA was found to be constitutively expressed in the head throughout the nymphal stages (Fig. 3*D*). For *Gb'CYP15A1*, a cytochrome P450 gene which is essential for JH biosynthesis (25), a slight change in its transcript levels was observed, and the highest transcript levels were detected in the 8th instar females (Fig. 3*F*). These results suggest that although *Gb'dpp* may play a role in regulating *Gb'jhamt* expression, *Gb'myo* appears to act as a rate-limiting factor in the *Gb'jhamt* expression pathway.

The spatial expression patterns of *Gb'myo*, *Gb'babo*, *Gb'dpp*, *Gb'tkv*, *Gb'jhamt*, and *Gb'CYP15A1* were also detected in the head with whole mount *in situ* hybridization. All of these genes were found to be predominantly expressed in the CA on day 3 of the 7th instar (Fig. 3 *G-M*). Similar results were obtained when the transcripts of these genes were detected in the CA by qPCR (Fig. 3 *N* and *O*). Thus, it appears that expression of *Gb'myo* in the CA correlates with the regulation of *Gb'jhamt* expression and JH biosynthesis.

Since both *Gb'myo* and *Gb'dpp* were found to be expressed in the CA, we investigated whether these genes are involved in the regulation of *Gb'jhamt* transcription. First, we confirmed that RNAi targeting of *Gb'myo* was effective in the heads of supernumerary nymphs (Fig. 4*A*). An increase in *Gb'jhamt* expression was also detected in the supernumerary instars on day 1 (3'-4th-4'), and these levels were significantly higher in these supernumerary nymphs on day 5 compared with the

undetectable levels of *Gb'jhamt* that characterized the controls (Fig. 4*B*). Consistent with the supernumerary molting of the nymphs that had received RNAi targeting *Gb'smox*, *Gb'jhamt* mRNA levels were up-regulated in 3' and 4th nymphs on day 5 (Fig. S5*B*). In contrast, no significant changes were observed in each of the supernumerary instars that expressed the *Gb'CYP15A1* transcript (Fig. 4*C*). When RNAi targeting *Gb'mad* (Fig. 4*D*), *Gb'medea* (Fig. S5*C*), or *Gb'gbb* (Fig. S5*D*) were injected into 3rd instars, *Gb'jhamt* transcript levels were lower in both the 4th and 6th instars on day 1; while no apparent effect on *Gb'CYP15A1* mRNA levels were observed in the *Gb'mad* depleted nymphs (Fig. 4*D*). Taken together, these results demonstrate that precocious metamorphosis in nymphs that received RNAi targeting *Gb'mad*, *Gb'medea*, or *Gb'gbb* derives from repression of *Gb'jhamt* expression, and they also suggest that up-regulation of *Gb'jhamt* in nymphs that received RNAi targeting *Gb'myo* or *Gb'smox* may depend on timely regulation of the *Gb*'Dpp/Gbb signaling pathway.

Following the injection of RNAi targeting *Gb'jhamt* into 3rd instar nymphs, precocious metamorphosis was observed, and these features were similar to those of RNAi depletion targeting *Gb'mad* (Fig. S6*A*). To examine whether the increase in *Gb'jhamt* expression caused by *Gb'myo*-targeted RNAi can be prevented with the knockdown of *Gb'mad* or *Gb'jhamt*, *Gb'myo* RNAi + *Gb'mad* RNAi and *Gb'myo* RNAi + *Gb'jhamt* RNAi were injected into 3rd instar nymphs (n = 9/12 and n = 15/16, respectively; Fig. S6 *B* and *C*). Changes in overall body size (Fig. S6 *D* and *E*) and relative transcript levels (Fig. S6*F*) were observed. Moreover, the supernumerary molting phenotype was rescued when *Gb'jhamt* was targeted for depletion. Thus, it

appears that supernumerary molts are caused by alterations in *Gb'jhamt* expression. However, the mechanisms underlying regulation of *Gb'jhamt* expression by *Gb*'Myo signaling are unknown.

In recent studies, the transcriptional repressor, Brinker (Brk), has been found to be a Dpp target that negatively regulates Dpp signaling in *Drosophila* (26, 27). Therefore, we examined the levels of *Gb'brk* mRNA in nymphs that received RNAi targeting genes related to *Gb*'Dpp/Gbb signaling (*Gb'mad*, *Gb'medea*, and *Gb'gbb*) or *Gb*'Myo signaling (*Gb'myo* and *Gb'smox*). In the former experiments, depletion of *Gb'mad* (Fig. 4*E*), *Gb'medea* (Fig. S5*C*), and *Gb'gbb* (Fig. S5*D*) resulted in an increase in *Gb'brk* mRNA levels in 4th and 6th instar nymphs on day 1. These results suggest that *Gb'brk* expression is negatively regulated by Gb'Dpp/Gbb signaling (Fig. 5*A*). Thus, we speculated that the transcriptional repressor *Gb*'Brk plays a role in negatively regulating Gb'Dpp/Gbb signaling, and it may regulate the repression of *Gb'jhamt*. To examine the latter possibility, RNAi targeting *Gb'brk* was injected into 3rd instar nymphs. While the control animals exhibited normal molting, the majority of the *Gb'brk* RNAi-treated nymphs arrested in the early developmental stages (25 out of 27). In addition, increased expression of *Gb'jhamt* mRNA was detected in the *Gb'brk* RNAi-treated nymphs during the 4th and 6th instars on day 1, yet no effect was observed on day 5 (Fig. 4*F*). These results suggest that *Gb*'Brk may be associated with negative regulation of *Gb'jhamt* (Fig. 5*B*). To investigate whether the reduction in *Gb'jhamt* expression in the *Gb'mad*-depleted nymphs was due to concomitant up-regulation of *Gb'brk* (Fig. 5*A*), dual RNAi targeting *Gb'mad* and *Gb'brk* were both injected into 3rd

instar nymphs. Subsequently, *Gb'mad* RNAi-dependent repression of *Gb'jhamt* that previously was observed in the 4th and 6th instars on day 1 was not rescued by depletion of *Gb'brk* (Fig. 4*F*). Thus, repression of *Gb'jhamt* in the nymphs that received RNAi targeting *Gb'mad* appeared to be independent of increased *Gb'brk* expression (Fig. 5*C*). Consequently, our results suggest that both an up-regulation of *Gb'jhamt* and a down-regulation of *Gb'brk* are controlled by the *Gb*'Dpp/Gbb/Mad signaling pathway (Fig. 5 *D* and *F*). While *Gb'brk* expression was markedly decreased on days 1 or 5 in the supernumerary nymphs (3' and 4th) with depletion of *Gb'myo* (Fig. 4*E*) and *Gb'smox* (Figs. S5*B* and 5*D*). Therefore, we propose that induction of *Gb'jhamt* and repression of *Gb'brk* that are dependent on the function of *Gb*'Mad may be blocked by *Gb*'Myo/Smox signaling (Fig. 5 *E* and *F*).

Previous studies have showed that *Daughters against dpp* (*dad*) is an inhibitory-Smad that is able to genetically antagonize Dpp signaling in *Drosophila* (28). Regulation of *dad* has also been reported to be effected by the function of Mad and Smox (29). In order to understand how *Gb*'Myo signaling prevents *Gb*'Dpp/Gbb signaling, we investigated whether the *Gb*'Dpp/Gbb and *Gb*'Myo signaling pathways are associated with expression of *Gb'dad*. When RNAi targeting *Gb'mad* was injected into 3rd instar nymphs, lower levels of *Gb'dad* mRNA were detected (Fig. S5*E*). In contrast, depletion of *Gb'smox* by RNAi had no effect on *Gb'dad* expression (Fig. S5*E*). These results suggest that *Gb'dad* may represent downstream target gene of *Gb*'Dpp/Gbb signaling, and *Gb*'Myo signaling may regulate the expression of *Gb'brk* and *Gb'jhamt* through the control of *Gb*'Dpp/Gbb signaling pathway (Fig. 5 *E* and *F*).

Overall, the results of the experiments performed suggest that *Gb*'Myo signaling suppresses *Gb*'*jhamt* expression that is induced by *Gb*'Dpp/Gbb signaling, and this leads to an inhibition of JH biosynthesis and an induction of metamorphosis.

Discussion

The results of the present study demonstrate that the TGF-β ligands, *Gb*'Dpp, *Gb*'Gbb, and *Gb*'Myo, regulate the synthesis of JH by regulating the expression of *Gb*'*jhamt* in the CA (Fig. 5*F*). As part of this process, transcription of the *jhamt* gene is controlled by the Dpp/Gbb/Tkv/Mad/Medea signaling pathway, while Myo/Babo/Smox signaling suppresses *jhamt* expression by controlling the Dpp/Gbb/Tkv/Mad/Medea signaling pathway. Expression of JHAMT in CA cells transforms JH acid into JH, and the latter is released into the hemolymph (Fig. 5*F*). We hypothesize that these regulatory mechanisms that determine the titre of JH are common in insects, including holometabola, for four reasons: 1) because the CA is a common endocrine gland which generates JH in insects; 2) *Gb*'Dpp functions in the CA similarly to *Dm*'Dpp in the CA of *Drosophila* (20); 3) *Dm*'Myoglianin (*Dm*'Myo), a homolog of *Gb*'Myo, is secreted by glial cells prior to metamorphosis to direct developmental neural remodeling (30); and 4) *Gb*'*myo* regulates final insect size via regulation of JH titre as observed in *Drosophila* (31).

However, RNAi treatment is not equivalent to genetic null, therefore it may not be possible to demonstrate the precise regulatory relationship between *smox*, *mad*, *brk*, and *jhamt* due to incomplete knockdown. In addition, RNAi knockdown occurs throughout the whole-body, and cannot be specifically targeted to the CA. Thus, the knockdown of these genes by RNAi may occur in other tissues. For example, *Gb*'*myo* and *Gb*'*dpp* are also expressed in the brains of *Gryllus bimaculatus* nymphs (Fig. S5*F*), and the mechanisms that regulate Dpp and Myo production in the brain remain to be

determined. It has been proposed that allatotropic and allatostatic peptides may play a role (9, 32). However, the phenotypes observed following targeting of the allatostatin-A type gene by RNAi (33) differ from the phenotypes generated by *Gb'myo* RNAi, yet are similar to the phenotypes obtained following up-regulation of JH. Thus, no significant relations between allatostatins and Myo have been identified. On the other hand, in the *Drosophila* prothoracic gland (PG), knockdown of *Activin*/*Babo*/*Smox* pathway causes developmental arrest prior to metamorphosis owing to control the ecdysone biosynthesis through the regulation of PTTH and insulin signaling pathways (34). Our results show that in *Gryllus bimaculatus* nymphs, *Gb'myo* is also expressed in the thorax 1 (prothorax) including the PG (Fig. S5*A*). Thus, *Gb*'Myo/Babo/Smox signaling may be independently associated with both JH and ecdysone biosynthesis. It should be noted, however, that as yet no connection between *Gb*'Myo and ecdysone biosynthesis has been established in this study. Finally, in mice, Myostatin/GDF8, a homolog of *Gb*'Myo, is a potent inhibitor of skeletal muscle growth (19), while another homolog of GDF11 has been reported to inhibit muscle formation (35, 36). Thus, GDF8/11 function might be an important regulator of adult muscle size. These GDF members are likely to be evolutionarily conserved as a body-size regulator among animals.

In conclusion, the present findings provide common regulatory mechanisms with TGF-β signaling to explain the endocrine control of invertebrate life cycles. We anticipate that further studies on regulation of the *Gb*'Myo signaling in the brain and PG will be of great interest.

Materials and Methods

Animals. All adult and nymph two-spotted *Gryllus bimaculatus* crickets were reared at 29 °C and 50% humidity under standard conditions as previously described (37).

Cloning. *Gryllus* genes related to Dpp/Myo-signaling genes were cloned by RT-PCR from 3rd instar nymph cDNA samples using the gene-specific primers listed in Table S1. A putative full length cDNA sequence containing the open reading frame (ORF) of *Gb'myo* (864 bp) was deposited in DDBJ (accession no. LC128665). RT-PCR was done as described in *SI Materials and Methods*.

RNA interference. The synthesis of RNAi was performed as described in *SI Materials and Methods*. Within 24 h after ecdysis, nymphs were injected with 20 μM RNAi in a volume of 0.2–0.5 μl into the ventral abdomen. RNAi targeting *DsRed2* was injected as a negative control. In the dual RNAi experiments, a combination of RNAi targeting *Gb'myo* and *Gb'jhamt*, *Gb'myo* and *Gb'mad*, *Gb'mad* and *Gb'brk*, or *Gb'dpp* and *Gb'gbb*, each with a final concentration of 20 μM, were injected.

Quantitative RT-PCR (qPCR). The qPCR primers used are listed in Table S2. RNA extraction, cDNA synthesis, and qPCR conditions are described in *SI Materials and Methods*.

***In situ* hybridization.** Digoxigenin (DIG)-labeled antisense RNA probes for *Gb'myo*,

Gb'babo, *Gb'dpp*, *Gb'tkv*, *Gb'jhamt*, and *Gb'CYP15A1* cDNA fragments obtained by RT-PCR were used for whole-mount *in situ* hybridization. *In situ* hybridization was performed as described in *SI Materials and Methods*.

JH extraction. *G. bimaculatus* nymphs were dissected and hemolymph (~5 ul per nymph) was extracted using methanol/isooctane (1:1, v/v) with 50ng fenoxycarb (Wako Pure Chemical Industries Ltd., Osaka, Japan) as an internal standard. Additional procedures for JH extraction are described in *SI Materials and Methods*.

LC-MS. An Ultra Performance Liquid Chromatography (UPLC)-LCT Premier system (Waters, Milford, MA, USA) was equipped with a 50 × 2.1 mm^2 C_{18} reverse phase column (ACQUITY UPLC BEH ODS-1.7 μm; Waters) that was protected by a VanGuard pre-column (Waters). Following the application of ACQUITY UPLC system, and was eluted with 100% methanol at a flow rate of 0.3 ml/min. MS analysis was performed as described in *SI Materials and Methods*.

Hormone treatment. A JH analogue, methoprene, was dissolved in ethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan) to a concentration of 100 μg/μl and then approximately 0.2 μl of this methoprene solution was injected into the ventral abdomen of newly molted 3rd or 5th instar nymphs (~20 μg of methoprene/nymph). The same volume of ethanol was injected as a control.

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Figure Legends

Fig. 1. Phenotypes observed after depletion of *Gb'mad* and *Gb'tkv* were achieved with RNAi in the nymph stage of *Gryllus bimaculatus*. (*A* and *B*) The effects of RNAi targeting *Gb'mad* or *Gb'tkv* in nymphs on day 1 of the 3rd instar. In each box, the control nymph is on the left and the RNAi-treated nymph is on the right. The instar and adult stages for each box are indicated at the bottom. The RNAi-treated nymphs remained small, yet underwent precocious adult metamorphosis at the 7th instar. (*C* and *D*) Body length (*C*) and weight (*D*) of the adults (male: ♂; and female: ♀) that developed following injections of RNAi targeting *DsRed2* (as a control) or *Gb'mad*. The data presented are the mean ± SD. *, $P < 0.05$ according to Student's *t-test*. (*E*) The wing pads (indicated with red asterisks) of the 6th instar *Gb'mad* RNAi nymphs exhibited abnormal growth and displayed an extended side. (*F*) The morphology of the ovipositor (indicated with arrows) in the *Gb'mad* RNAi 6th instar nymphs was smaller than that of the control nymphs (Figs. 2*O* and S4*J*). (*G*) Precocious adults were produced following the injection of RNAi targeting *Gb'mad*. The wings of these adults were significantly smaller and were wrinkled. (*H*) The ovipositors of the adults produced following the injection of RNAi targeting *Gb'mad* were cleaved at the tip and they became abnormally short. Scale bars: 10 mm in *A* and *B*; 2 mm in *E*, *G*, and *H*; and 1 mm in *F*.

Fig. 2. Phenotypes observed after depletion of *Gb'myo* was achieved with RNAi in *Gryllus bimaculatus*. (*A* and *B*) RNAi targeting *DsRed2* (control) or *Gb'myo* were

injected into 3rd instars on day 1. Morphological variations during supernumerary molts (3'-3''-4th-4'-4'') and during metamorphosis were subsequently observed in *A* and *B*, respectively. In *A*, the control nymph is on the left and the RNAi-treated nymph is on the right in each box. The instar and adult stages for each box are indicated at the bottom (male: ♂; and female: ♀). (*C-E*) Lateral views of the 3rd (*C*), 4th (*D*), and 5th (*E*) instar nymphs that were injected with RNAi targeting *DsRed2* on day 1 of the 3rd instar. The red lines indicate the contours of the wing pads (indicated with asterisks). T1-3; thorax 1-3. (*F*) Dorsal view of the wing pads (indicated with asterisks) in a representative 6th instar nymph that was injected with RNAi targeting *DsRed2* on day 1 of the 3rd instar. (*G-J*) Lateral views of supernumerary 3' (*G*), 3'' (*H*), 4' (*I*), and 4'' (*J*) instar nymphs that were injected with RNAi targeting *Gb'myo* on day 1 of the 3rd instar. (*K*) Dorsal view of a representative supernumerary 4'' instar nymph that was injected with RNAi targeting *Gb'myo* on day 1 of the 3rd instar. (*L-O*) Ventral views of 3rd (*L*), 4th (*M*), 5th (*N*), and 6th (*O*) instar nymphs that were injected with RNAi targeting *DsRed2* on day 1 of the 3rd instar. Morphological alterations in the ovipositors (indicated with arrows) at the abdomen 8 (Abd8; indicated with arrowheads) were observed. (*P-S*) Ventral views of supernumerary 3' (*P*), 3'' (*Q*), 4' (*R*), and 4'' (*S*) instar nymphs that were injected with RNAi targeting *Gb'myo* on day 1 of the 3rd instar. (*T* and *U*) Body length (*T*) and weight (*U*) of nymphs and adults that were treated with RNAi targeting *DsRed2* (black) or *Gb'myo* (red). Weeks post-injection (w) were indicated in the X-axis. The data presented are the mean ± SD. Scale bars: 10 mm in *A*

and *B*; 0.5 mm in *C*, and *L*-*O*; and 2 mm in *F* and *K*.

Fig. 3. Expression profiles of *Gb'myo*, *Gb'dpp*, *Gb'jhamt*, and *Gb'CYP15A1* transcripts in *Gryllus bimaculatus* during development, and the effect of RNAi targeting *Gb'myo* and *Gb'mad* on the hemolymph titre of JH. (*A*) Developmental changes in JH III titre in the hemolymph of male (dotted line) and female (solid line) nymphs that were collected from 4th to 8th instars. (*B*) JH III titre measurements in the hemolymph of nymphs treated with RNAi targeting *Gb'myo* (red) or *Gb'mad* (blue) in the 3rd instar. Asterisks represent significant differences between control and RNAi nymphs: *, $P < 0.05$ according to Student's *t*-test. (*C*-*F*) Temporal expression of *Gb'myo* (*C*), *Gb'dpp* (*D*), *Gb'jhamt* (*E*), and *Gb'CYP15A1* (*F*) as detected in qPCR analyses of nymph heads. Relative fold changes in the mRNA levels were plotted, and the average expression level in the heads on day 1 of the 3rd instar (D1 3rd) was set to 1. The mRNA levels were also normalized to *Gb'β-actin* mRNA levels. Developmental stages were defined as days (D) after molting. Nymphs were unsexed during the 3rd to 5th instars and were sexed during the 6th to 8th instars and the adult (ad) stage (male data: dotted line; female data: solid line). The data presented are the mean ± SD. (*G*-*M*) Expression levels of *Gb'myo* (*G*), *Gb'baboon* (*H*), *Gb'dpp* (*I*), *Gb'tkv* (*J*), *Gb'jhamt* (*K*), and *Gb'CYP15A1* (*L*) in the corpus allatum-corpus cardiacum (CA-CC) complex on day 3 of the 7th instar were examined by whole-mount *in situ* hybridization. A control experiment using the *Gb'myo* sense probe is shown in *M*. (*N* and *O*) Expression levels of *Gb'myo*, *Gb'dpp*, *Gb'jhamt*, and *Gb'CYP15A1* as detected in qPCR analyses of RNA

samples collected from the CA (*N*) and CC (*O*) of 7th instar nymphs. The expression level of *Gb'jhamt* was set to 1. The data presented are the mean ± SD.

Fig. 4. The effects of RNAi-mediated depletion of *Gb'myo* and *Gb'mad* on the expression of *Gb'jhamt*, *Gb'CYP15A1*, and *Gb'brk*. (*A-C*) RNAi targeting *DsRed2* control or *Gb'myo* were injected on day 1 of the 3rd instar. Transcript levels of *Gb'myo* (*A*), *Gb'jhamt* (*B*), and *Gb'CYP15A1* (*C*) were subsequently determined on days 1 and 5 in the heads of the supernumerary 3rd, 3', 4th, and 4' instars. The transcript levels determined on day 1 of the 3rd instar control nymphs (D1 3rd) for panels *A-C* were set to 1. The data presented are the mean ± SD. (*D*) Transcript levels of *Gb'mad*, *Gb'jhamt*, and *Gb'CYP15A1* were also determined on day 1 of the 4th and 6th instars following the injection of RNAi targeting *Gb'mad*. The transcript levels of these genes in control nymphs on day 1 of the 4th instar (D1 4th) were set to 1. The data presented are the mean ± SD. (*E*) *Gb'brk* mRNA levels in the heads of 4th (3') and 6th (4th) instar nymphs on days 1 and 5 after the injection of RNAi targeting *Gb'myo* (red), and on day 1 for the 4th and 6th instar nymphs that received RNAi targeting *Gb'mad* (blue). The transcript levels of both sets of control nymphs on day 1 of the 4th instar were set to 1. The data presented are the mean ± SD. (*F*) Following RNAi-mediated depletion of *Gb'brk* (green) or *Gb'mad* + *Gb'brk* (yellow) in the 3rd instar transcript levels of *Gb'brk* or *Gb'jhamt* were measured on days 1 and 5 of the 4th and 6th instars as indicated. The transcript levels measured on day 5 (D5 4th) or day 1 (D1 4th) of the control 4th instar nymphs were set to 1, respectively. The data presented are the mean ± SD. Asterisks in

A, *B*, and *D-F* represent significant differences between the control and RNAi nymphs. n.s., not significant; *, P < 0.05; **, P < 0.005; ***, P < 0.001 according to Student's *t*-test.

Fig. 5. Regulation of *Gb'jhamt* expression. (*A-E*) Schematic diagrams of *Gb'brk* and *Gb'jhamt* transcriptional regulation based on the results obtained from experiments that involved the targeting of *Gb'mad* (*A* and *E*), *Gb'brk* (*B*), *Gb'mad* + *Gb'brk* (*C*), and *Gb'smox* (*D*) genes by RNAi. Gray colors denote gene depletion and transcriptional regulatory effects by RNAi. Red arrows indicate the down- and up-regulation of target gene expression. (*F*) This diagram depicts the function of Dpp/Gbb (blue) and Myo (pink) signaling pathways in the regulation of *jhamt* expression and JH action.